

TRANSLATOR'S DECLARATION

I, Janet Hope, BSc (Hons.), MIL., MITI., translator to Messrs. Taylor and Meyer of 20 Kingsmead Road, London, SW2 3JD, Great Britain, verify that I know well both the German and the English language, that I have prepared the attached English translation of 46 pages of a German Patent application in the German language with the title:

Neue für das otsA-Gen kodierende Nukleotidsequenzen

identified by the code number 010037 BT / IP at the upper left of each page and corresponding to client/matter number of the law firm of

and that the attached English translation of this document is a true and correct translation of the document attached thereto to the best of my knowledge and belief.

I further declare that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 USC 1001, and that such false statements may jeopardize the validity of this document.

By: 

Date: 18<sup>TH</sup> MAY 2006

# FEDERAL REPUBLIC OF GERMANY



## Certificate of Priority for Filing of a Patent Application

**Filing number:** 101 10 760.9

**Filing date:** 7<sup>th</sup> March 2001

**Applicant/Proprietor:** Degussa AG, Düsseldorf/Germany

**Title:** New nucleotide sequences which code for the otsA gene

**Priority:** 30.01.2001 DE 101 03 873.9

**IPC:** C 21 N, C 12 Q, C 07 H

**The attached papers are a true and accurate reproduction of the original documents for this patent application.**

Munich, 22<sup>nd</sup> November 2001  
**On behalf of the President of the German  
Patent and Trade Mark Office**

*(signature)*

Wallner

**New nucleotide sequences which code for the otsA gene**

The invention provides nucleotide sequences from coryneform bacteria which code for the otsA gene and a process for the fermentative preparation of amino acids using bacteria in  
5 which the otsA gene is attenuated.

**Prior art**

L-Amino acids, in particular L-lysine, are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal  
10 nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve  
15 the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to  
20 the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these  
25 microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce amino acids are obtained in this manner.

Methods of the recombinant DNA technique have also been  
30 employed for some years for improving the strain of Corynebacterium strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

### Object of the invention

The inventors had the object of providing new measures for improved fermentative preparation of amino acids.

### Description of the invention

5 Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-  
10 isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Lysine is particularly preferred.

When L-lysine or lysine are mentioned in the following, not only the bases but also the salts, such as e.g. lysine  
15 monohydrochloride or lysine sulfate, are meant by this.

The invention provides an isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the otsA gene, chosen from the group consisting of

- 20 a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide which  
25 comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b),

- d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

the polypeptide preferably having the activity of trehalose 6-phosphate synthase.

The invention also provides the abovementioned polynucleotide, this preferably being a DNA which is capable of replication, comprising:

- (i) the nucleotide sequence, shown in SEQ ID No.1, or
- 10 (ii) at least one sequence which corresponds to sequence (i) within the degeneracy of the genetic code, or
- (iii) at least one sequence which hybridizes with the sequences complementary to sequences (i) or (ii), and optionally
- 15 (iv) sense mutations of neutral function in (i) which do not modify the activity of the protein/polypeptide.

Finally, the invention also provides polynucleotides chosen from the group consisting of

- a) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 1 and 883,
- 20 b) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 884 and 2338,
- 25 c) polynucleotides comprising at least 15 successive nucleotides chosen from the nucleotide sequence of SEQ ID No. 1 between positions 2339 and 3010.

The invention also provides:

a polynucleotide, in particular DNA, which is capable of replication and comprises the nucleotide sequence as shown in SEQ ID No.1;

5 a polynucleotide which codes for a polypeptide which comprises the amino acid sequence as shown in SEQ ID No. 2;

a vector containing parts of the polynucleotide according to the invention, but at least 15 successive nucleotides of the sequence claimed,

10 and coryneform bacteria in which the otsA gene is attenuated, in particular by an insertion or deletion.

The invention also provides polynucleotides, which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a  
15 corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotide according to the invention according to SEQ ID No.1 or a fragment thereof, and isolation of the polynucleotide  
20 sequence mentioned.

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for  
25 trehalose 6-phosphate synthase or to isolate those nucleic acids or polynucleotides or genes which have a high similarity with the sequence of the otsA gene. They are also suitable for incorporation into so-called "arrays", "micro arrays" or "DNA chips" in order to detect and  
30 determine the corresponding polynucleotides.

Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for trehalose 6-

phosphate synthase can be prepared by the polymerase chain reaction (PCR).

Such oligonucleotides which serve as probes or primers comprise at least 25, 26, 27, 28, 29 or 30, preferably at least 20, 21, 22, 23 or 24, very particularly preferably at least 15, 16, 17, 18 or 19 successive nucleotides.

Oligonucleotides with a length of at least 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 or at least 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides are also suitable.

Oligonucleotides with a length of at least 100, 150, 200, 250 or 300 nucleotides are optionally also suitable.

"Isolated" means separated out of its natural environment.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90% and very particularly preferably at least 91%, 93%, 95%, 97% or 99% identical to the polynucleotide according to SEQ ID No. 1 or a fragment prepared therefrom.

"Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include a polypeptide according to SEQ ID No. 2, in particular those with the biological activity of trehalose 6-phosphate synthase, and also those which are at least 70% to 80%, preferably at least 81% to 85%, particularly preferably at least 86% to 90% and very particularly preferably at least

91%, 93%, 95%, 97% or 99% identical to the polypeptide according to SEQ ID No. 2 and have the activity mentioned.

The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine using coryneform bacteria which in particular already produce amino acids and in which the nucleotide sequences which code for the otsA gene are attenuated, in particular eliminated or expressed at a low level.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme or protein with a low activity or inactivates the corresponding gene or enzyme (protein) and optionally combining these measures.

The microorganisms provided by the present invention can prepare amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains



Corynebacterium glutamicum ATCC13032  
Corynebacterium acetoglutamicum ATCC15806  
Corynebacterium acetoacidophilum ATCC13870  
Corynebacterium melassecola ATCC17965  
5 Corynebacterium thermoaminogenes FERM BP-1539  
Brevibacterium flavum ATCC14067  
Brevibacterium lactofermentum ATCC13869 and  
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants or strains prepared  
10 therefrom, such as, for example, the L-lysine-producing  
strains

Corynebacterium glutamicum FERM-P 1709  
Brevibacterium flavum FERM-P 1708  
Brevibacterium lactofermentum FERM-P 1712  
15 Corynebacterium glutamicum FERM-P 6463  
Corynebacterium glutamicum FERM-P 6464  
Corynebacterium glutamicum DM58-1  
Corynebacterium glutamicum DG52-5  
Corynebacterium glutamicum DSM5715 and  
20 Corynebacterium glutamicum DSM12866.

The new otsA gene from C. glutamicum which codes for the  
enzyme trehalose 6-phosphate synthase (EC 2.4.1.15) has  
been isolated.

To isolate the otsA gene or also other genes of C.  
25 glutamicum, a gene library of this microorganism is first  
set up in Escherichia coli (E. coli). The setting up of  
gene libraries is described in generally known textbooks  
and handbooks. The textbook by Winnacker: Gene und Klone,  
Eine Einführung in die Gentechnologie (Verlag Chemie,  
30 Weinheim, Germany, 1990), or the handbook by Sambrook et  
al.: Molecular Cloning, A Laboratory Manual (Cold Spring  
Harbor Laboratory Press, 1989) may be mentioned as an  
example. A well-known gene library is that of the E. coli  
K-12 strain W3110 set up in  $\lambda$  vectors by Kohara et al.

(Cell 50, 495-508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, 5 Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326 (1992)) in turn describe a gene library of *C. glutamicum* 10 ATCC13032 using the cosmid pH79 (Hohn and Collins, 1980, Gene 11, 291-298).

To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, 1979, Life Sciences, 25, 807-818) or pUC9 (Vieira et al., 15 1982, Gene, 19:259-268). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-defective, such as, for example, the strain DH5 $\alpha$ mc<sup>r</sup>, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 20 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids or other  $\lambda$  vectors can then in turn be subcloned and subsequently sequenced in the usual vectors which are suitable for DNA sequencing, such as is described e. g. by Sanger et al. (Proceedings of the National Academy 25 of Sciences of the United States of America, 74:5463-5467, 1977).

The resulting DNA sequences can then be investigated with known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217- 30 232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

The new DNA sequence of *C. glutamicum* which codes for the *otsA* gene and which, as SEQ ID No. 1, is a constituent of

the present invention has been found. The amino acid sequence of the corresponding protein has furthermore been derived from the present DNA sequence by the methods described above. The resulting amino acid sequence of the  
5   otsA gene product is shown in SEQ ID No. 2. It is known that enzymes endogenous in the host can split off the N-terminal amino acid methionine or formylmethionine of the protein formed.

Coding DNA sequences which result from SEQ ID No. 1 by the  
10   degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or  
15   of aspartic acid for glutamic acid in proteins, are furthermore known among experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. Such mutations are also called, inter alia, neutral substitutions. It is  
20   furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in  
25   O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2  
30   are also a constituent of the invention.

In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers which result  
35   from SEQ ID No. 1 are a constituent of the invention. Such

oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of hybridization can be found by the expert, inter alia, in the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology 41: 255-260 (1991)). The hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i. e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington, UK, 1996).

A 5x SSC buffer at a temperature of approx. 50°C - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim, Mannheim, Germany, 1995), a temperature of approx. 50°C - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% or at least 96% to 99% identical to the sequence of the probe employed can be isolated by increasing the hybridization temperature

stepwise from 50°C to 68°C in steps of approx. 1 - 2°C. It is also possible to isolate polynucleotide fragments which are completely identical to the sequence of the probe employed. Further instructions on hybridization are  
5 obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics GmbH, Mannheim, Germany, Catalogue No. 1603558).

Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by  
10 the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino  
15 acids in an improved manner after attenuation of the otsA gene.

To achieve an attenuation, either the expression of the otsA gene or the catalytic/regulatory properties of the enzyme protein can be reduced or eliminated. The two  
20 measures can optionally be combined.

The reduction in gene expression can take place by suitable culturing or by genetic modification (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes,  
25 activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. The expert can find information on this e.g. in the patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss  
30 (Nucleic Acids Research 26: 3548 (1998)), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)), in Pátek et al. (Microbiology 142: 1297 (1996)), Vasicova et al. (Journal of Bacteriology 181: 6188 (1999)) and in known textbooks of genetics and molecular biology, such as

e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

5 Mutations which lead to a change or reduction in the catalytic properties of enzyme proteins are known from the prior art; examples which may be mentioned are the works by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and  
10 Biochemistry 61: 1760-1762 (1997)) and Möckel ("Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms", Reports from the Jülich Research Centre, Jül-2906, ISSN09442952, Jülich, Germany, 1994). Summarizing  
15 descriptions can be found in known textbooks of genetics and molecular biology, such as e.g. that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

Possible mutations are transitions, transversions,  
20 insertions and deletions. Depending on the effect of the amino acid exchange on the enzyme activity, "missense mutations" or "nonsense mutations" are referred to. Insertions or deletions of at least one base pair (bp) in a gene lead to frame shift mutations, as a consequence of  
25 which incorrect amino acids are incorporated or translation is interrupted prematurely. Deletions of several codons typically lead to a complete loss of the enzyme activity. Instructions on generation of such mutations are prior art and can be found in known textbooks of genetics and  
30 molecular biology, such as e.g. the textbook by Knippers ("Molekulare Genetik", 6th edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer  
35 Verlag, Stuttgart, 1986).

A common method of mutating genes of *C. glutamicum* is the method of "gene disruption" and "gene replacement" described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

5 In the method of gene disruption a central part of the coding region of the gene of interest is cloned in a plasmid vector which can replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791  
10 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-  
15 84; US Patent 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516). The plasmid vector which contains the central part of the coding region  
20 of the gene is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for  
25 example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross-over" event,  
30 the coding region of the gene in question is interrupted by the vector sequence and two incomplete alleles are obtained, one lacking the 3' end and one lacking the 5' end. This method has been used, for example, by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580  
35 (1994)) to eliminate the *recA* gene of *C. glutamicum*.

In the method of "gene replacement", a mutation, such as e.g. a deletion, insertion or base exchange, is established in vitro in the gene of interest. The allele prepared is in turn cloned in a vector which is not replicative for *C. glutamicum* and this is then transferred into the desired host of *C. glutamicum* by transformation or conjugation. After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second "cross-over" event which effects excision in the target gene or in the target sequence, the incorporation of the mutation or of the allele is achieved. This method was used, for example, by Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998)) to eliminate the *pyc* gene of *C. glutamicum* by a deletion.

15 A deletion, insertion or a base exchange can be incorporated into the *otsA* gene in this manner.

In addition, it may be advantageous for the production of L-amino acids to enhance, in particular over-express, one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the attenuation of the *otsA* gene.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or of the genes or alleles, using a potent promoter or using a gene or allele which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

Thus, for the preparation of L-lysine, in addition to the attenuation of the *otsA* gene at the same time one or more of the genes chosen from the group consisting of



- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
  - the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
  - the eno gene which codes for enolase (DE: 19947791.4),
  - the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
  - the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
  - the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
  - the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
  - the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
  - the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-B-0387527; EP-A-0699759; WO 00/63388),
  - the lysE gene which codes for lysine export (DE-A-195 48 222),
  - the zwal gene which codes for the Zwal protein (DE: 19959328.0, DSM 13115)
- can be enhanced, in particular over-expressed.

It may be furthermore advantageous for the production of L-lysine, in addition to the attenuation of the otsA gene, at the same time for one or more of the genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- 5 • the poxB gene which codes for pyruvate oxidase (DE:1995 1975.7, DSM 13114),
- the zwa2 gene which codes for the Zwa2 protein (DE: 19959327.2, DSM 13113),
- 10 • the fda gene which codes for fructose 1,6-bisphosphate aldolase (Accession No. X17313; von der Osten et al., Molecular Microbiology 3 (11), 1625-1637 (1989)),
- the hom gene which codes for homoserine dehydrogenase (EP-A-0131171),
- 15 • the thrB gene which codes for homoserine kinase (Peoples, O.W., et al., Molecular Microbiology 2 (1988): 63 - 72) and
- the panD gene which codes for aspartate decarboxylase (EP-A-1006192) and

20 to be attenuated, in particular for the expression thereof to be reduced.

The attenuation of homoserine dehydrogenase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-valine for L-alanine, L-glycine or L-leucine in position 59 of the enzyme protein, by  
25 exchange of L-valine by L-isoleucine, L-valine or L-leucine in position 104 of the enzyme protein and/or by exchange of L-asparagine by L-threonine or L-serine in position 118 of the enzyme protein.

The attenuation of homoserine kinase can also be achieved,  
30 inter alia, by amino acid exchanges, such as, for example,

by exchange of L-alanine for L-valine, L-glycine or L-leucine in position 133 of the enzyme protein and/or by exchange of L-proline by L-threonine, L-isoleucine or L-serine in position 138 of the enzyme protein.

- 5 The attenuation of aspartate decarboxylase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchanges of L-alanine for L-glycine, L-valine or L-isoleucine in position 36 of the enzyme protein.
- 10 In addition to the attenuation of the otsA gene it may furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta,
- 15 Vanek (eds.), Academic Press, London, UK, 1982).

The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed

20 batch process (repetitive feed process) for the purpose of production of L-amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart,

25 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions

30 of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture.

Antifoams, such as, for example, fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as, for

example, antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as, for example, air, are introduced into the culture. The  
5 temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.

Methods for the determination of L-amino acids are known  
10 from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography with subsequent ninhydrin derivatization, or it can be carried out by reversed phase HPLC, for example  
15 as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention is used for the fermentative preparation of amino acids, in particular L-lysine.

20 The following microorganism was deposited on 08.02.2001 as a pure culture at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- 25 • *Corynebacterium glutamicum* strain DSM5715ΔotsA as DSM 14041.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The isolation of plasmid DNA from *Escherichia coli* and all  
30 techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA).

Methods for transformation of *Escherichia coli* are also described in this handbook.

The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et al.

#### Example 1

Preparation of a genomic cosmid gene library from *C. glutamicum* ATCC 13032

Chromosomal DNA from *C. glutamicum* ATCC 13032 is isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme *Sau3AI* (Amersham Pharmacia, Freiburg, Germany, Product Description *Sau3AI*, Code no. 27-0913-02). The DNA fragments are dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987), Proceedings of the National Academy of Sciences, USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vector Kit, Code no. 251301) is cleaved with the restriction enzyme *XbaI* (Amersham Pharmacia, Freiburg, Germany, Product Description *XbaI*, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

The cosmid DNA is then cleaved with the restriction enzyme *BamHI* (Amersham Pharmacia, Freiburg, Germany, Product Description *BamHI*, Code no. 27-0868-04). The cosmid DNA treated in this manner is mixed with the treated ATCC13032 DNA and the batch is treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture is then packed in phages with the aid of Gigapack II XL Packing

Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acids Res. 16:1563-1575) the cells are taken  
5 up in 10 mM MgSO<sub>4</sub> and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library are carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox,  
10 1955, Virology, 1:190) + 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones are selected.

#### Example 2

##### Isolation and sequencing of the otsA gene

15 The cosmid DNA of an individual colony is isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product  
20 Description Sau3AI, Product No. 27-0913-02). The DNA fragments are dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid  
25 fragments in the size range of 1500 to 2000 bp are isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, The Netherlands, Product Description  
30 Zero Background Cloning Kit, Product No. K2500-01) is cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid

fragments in the sequencing vector pZero-1 is carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture is then electroporated (Tauch et al. 1994, FEMS Microbiol. Letters, 123:343-7) into the E. coli strain DH5 $\alpha$ mcr (Grant, 1990, Proceedings of the National Academy of Sciences, U.S.A., 87:4645-4649). Letters, 123:343-7) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clones is carried out with a Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing is carried out by the dideoxy chain-stopping method of Sanger et al. (1977, Proceedings of the National Academies of Sciences, U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction are carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained are then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives are assembled to a continuous contig. The computer-assisted coding region analysis is prepared with the XNIP program (Staden, 1986, Nucleic Acids Research 14:217-231).

The resulting nucleotide sequence is shown in SEQ ID No. 1. Analysis of the nucleotide sequence shows an open reading



frame of 1485 bp, which is called the otsA gene. The otsA gene codes for a polypeptide of 485 amino acids.

### Example 3

Construction of the vector pK19mobsacB $\Delta$ otsA for deletion of  
5 the otsA gene

#### 3.1. Cloning of the otsA gene in the vector pUC18

For this, chromosomal DNA is isolated from the strain ATCC13032 by the method of Tauch et al. (1995, Plasmid 33:168-179). On the basis of the sequence of the otsA gene  
10 known for C.glutamicum from example 2, the oligonucleotides described below are chosen for generation of the otsA deletion allele (see also SEQ ID No. 3 and SEQ ID No.4):

otsA fwd:

5'-CAC CTA TTC TAA GGA CTT CTT CGA -3'

15 otsA rev:

5'-ACC AAC CAG GTG GAA TCT GTC A-3'

The primers shown are synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR protocols. A  
20 Guide to Methods and Applications, 1990, Academic Press) with the Taq-polymerase from Boehringer Mannheim (Germany, Product Description Taq DNA polymerase, Product No. 1 146 165). With the aid of the polymerase chain reaction, the primers allow amplification of a DNA fragment approx.  
25 1.8 kb in size. The product amplified in this way is tested electrophoretically in a 0.8% agarose gel.

The PCR product obtained is then cloned in the vector pUC18 (Amersham Pharmacia Biotech, Cat. No. 27-4949-01) with the Sure Clone Ligation Kit from Amersham Pharmacia Biotech  
30 (Freiburg, Germany) in accordance with the manufacturer's

instructions. The vector pUC18 was linearized beforehand with the restriction enzyme SmaI.

The E.coli strain DH5 $\alpha$ mc<sup>r</sup> (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) is then  
5 electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, IRL-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation  
10 batch on LB agar (Sambrock et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which has been supplemented with 25 mg/l ampicillin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by  
15 restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid is called pUC18otsA and is shown in figure 1.

### 3.2. Introduction of a deletion into the cloned otsA gene fragment

20 From the plasmid pUC18otsA, a fragment 213 bp in size is excised from the central region of the otsA gene with the restriction enzymes PflMI and HpaI. The 3' overhangs formed from the PflMI digestion are removed with T4 DNA polymerase (Amersham Pharmacia Biotech, Freiburg, Germany; Code No.  
25 E2040Y) in accordance with the manufacturer's instructions. The residual vector is subjected to autoligation with T4 DNA ligase (Amersham Pharmacia Biotech, Freiburg, Germany; Code No. 27-0870-04) in accordance with the manufacturer's instructions and the ligation batch is electroporated  
30 (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) in the E. coli strain DH5 $\alpha$  (Hanahan, In: DNA cloning. A Practical Approach. Vol. I, IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Lennox,

1955, Virology, 1:190) with 25 mg/l ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, 5 Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and cleaved with the restriction enzyme EcoRI to check the plasmid by subsequent agarose gel electrophoresis. The resulting plasmid is called pUC18 $\Delta$ otsA.

### 10 3.3. Construction of the replacement vector pK19mobsac $\Delta$ otsA

The otsA deletion allele is isolated by complete cleavage of the vector pUC18 $\Delta$ otsA, obtained in example 3.2, with the restriction enzymes SacI/XbaI. After separation in an agarose gel (0.8%), the otsA $\Delta$  fragment approx. 1.6 kb in 15 size is isolated from the agarose gel with the aid of the Qiagenquick Gel Extraction Kit (Qiagen, Hilden, Germany). The 5' and 3' overhangs formed by the restriction digestion are removed with T4 DNA polymerase (Amersham Pharmacia Biotech, Freiburg, Germany; Code No. E2040Y) in accordance 20 with the manufacturer's instructions.

The otsA deletion allele treated in this way is employed for ligation with the mobilizable cloning vector pK19mobsacB (Schäfer et al., Gene 14: 69-73 (1994)). This was cleaved open beforehand with the restriction enzyme 25 SmaI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product No. 1758250). The vector DNA is mixed with the otsA deletion allele and the mixture is treated with T4 DNA ligase (Amersham- Pharmacia, Freiburg, Germany).

30 The E.coli strain DH5 $\alpha$ mc<sup>r</sup> (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) is then electroporated with the ligation batch (Hanahan, In. DNA cloning. A practical approach. Vol.1. ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-

carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989), which has been supplemented with 25 mg/l kanamycin.

- 5 Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and the cloned *otsA* deletion allele is verified by means of sequencing by MWG Biotech (Ebersberg, Germany). The plasmid is called pK19mobsacB $\Delta$ *otsA* and is shown in figure 2.

10 Example 4

Deltion mutagenesis of the *otsA* gene in the *C. glutamicum* strain DSM 5715

- The vector pK19mobsacB $\Delta$ *otsA* mentioned in example 3.3 is electroporated by the electroporation method of Tauch et al. (1989 FEMS Microbiology Letters 123: 343-347) in  
15 *Corynebacterium glutamicum* DSM5715. The vector cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome. Selection of clones with integrated pK19mobsacB $\Delta$ *otsA* takes  
20 place by plating out the electroporation batch on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which was supplemented with 15 mg/l kanamycin. Incubation is carried  
25 out for 2 days at 33°C.

- Clones which have grown on are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 16 hours at 33°C. To achieve excision of the plasmid together with the complete chromosomal copy of the *otsA* gene, the clones are  
30 then grown on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup> Ed., Cold Spring Harbor, New York, 1989) with 10% sucrose. The plasmid pK19mobsacB contains a copy of the *sacB* gene, which converts sucrose into levan

sucrase, which is toxic to *C. glutamicum*. Only those clones in which the pK19mobsacB $\Delta$ otsA integrated has been excised again therefore grow on LB agar with sucrose. In the excision, together with the plasmid either the complete  
5 chromosomal copy of the otsA gene can be excised, or the incomplete copy with the internal deletion. To demonstrate that the incomplete copy of otsA has remained in the chromosome, the plasmid pK9mobsacB $\Delta$ otsA is marked by the method of "The DIG System Users Guide for Filter  
10 Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) using the Dig hybridization kit from Boehringer. Chromosomal DNA of a potential deletion mutant is isolated by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)) and in each case cleaved with the  
15 restriction enzymes EcoRI and PstI in separate batches. The fragments formed are separated by agarose gel electrophoresis and hybridized at 68°C with the Dig hybridization kit from Boehringer. With the aid of the fragments formed, it can be shown that the strain DSM5715  
20 has lost its complete copy of the otsA gene and instead has only the copy with the deletion.

The strain is called *C. glutamicum* DSM5715 $\Delta$ otsA and deposited as a pure culture on 08.02.2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ =  
25 German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as DSM 14041 in accordance with the Budapest Treaty.

#### Example 5

##### Preparation of lysine

30 The *C. glutamicum* strain DSM5715 $\Delta$ otsA obtained in example 4 is cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant is determined.

For this, the strain is first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with kanamycin (25 mg/l) for 24 hours at 33°C. Starting from this agar plate culture, a preculture is seeded (10 ml  
5 medium in a 100 ml conical flask). The complete medium CgIII is used as the medium for the preculture.

#### Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Sucrose (autoclaved separately)	2% (w/v)

The pH is brought to pH 7.4

Kanamycin (25 mg/l) is added to this. The preculture is incubated for 16 hours at 33°C at 240 rpm on a shaking  
10 machine. A main culture is seeded from this preculture such that the initial OD (660 nm) of the main culture is 0.1 OD. The medium Cg XII (Keilhauer et al. 1993, Journal of Bacteriology 175:5595-5603) with addition of 0.1 g/l leucine is used for the main culture.

#### 15 Medium Cg XII

MOPS (morpholinopropanesulfonic acid)	42 g/l
Urea	5 g/l
Salts:	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20 g/l
KH <sub>2</sub> PO <sub>4</sub>	1 g/l
K <sub>2</sub> HPO <sub>4</sub>	1 g/l

MgSO <sub>4</sub> * 7 H <sub>2</sub> O	0.25 g/l
CaCl <sub>2</sub> * 2 H <sub>2</sub> O	10 mg/l
FeSO <sub>4</sub> * 7 H <sub>2</sub> O	10 mg/l
MnSO <sub>4</sub> * H <sub>2</sub> O	10 mg/l
ZnSO <sub>4</sub> * 7 H <sub>2</sub> O	1 mg/l
CuSO <sub>4</sub>	0.2 mg/l
NiCl <sub>2</sub>	0.02 mg/l
Biotin (sterile-filtered)	0.3 mg/l
Leucine (sterile-filtered)	0.1 g/l
Protocatechuic acid (sterile-filtered)	0.03 mg/l
Sucrose (autoclaved separately)	6% (w/v)

MOPS and the salt solution are brought to pH 7 and autoclaved. The sterile substrate and vitamin solutions are then added.

5 Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) is added. Culturing is carried out at 33°C and 80% atmospheric humidity.

10 After 73 hours, the OD is determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed is determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in table 1.

Table 1

Strain	OD (660 nm)	Lysine HCl mM
DSM5715	8.2	39
DSM5715 $\Delta$ otsA	8.4	52

The following figures are attached:

- Figure 1: Plasmid pUC18otsA
- 5 • Figure 2: Plasmid pK19mobsacB $\Delta$ otsA

The base pair numbers stated are approx. values obtained in the context of reproducibility.

The abbreviations and designations used have the following meaning:

lacZ': 5' terminus of the lacZ $\alpha$  gene fragment  
 'lacZ: 3' terminus of the lacZ $\alpha$  gene fragment  
 otsA: otsA Gene  
 Amp: Ampicillin resistance gene  
 oriV: ColE1-similar origin from pMB1  
 RP4mob: RP4 mobilization site  
 Kan: Kanamycin resistance gene  
 otsA': 5' terminal fragment of the pck gene  
 ''otsA: 3' terminal fragment of the pck gene  
 sacB: The sacB gene which codes for the protein



levan sucrose

EcoRI: Cleavage site of the restriction enzyme EcoRI

HpaI: Cleavage site of the restriction enzyme HpaI

PflMI: Cleavage site of the restriction enzyme PflMI

PstI: Cleavage site of the restriction enzyme PstI

SacI: Cleavage site of the restriction enzyme SacI

XbaI: Cleavage site of the restriction enzyme XbaI

## SEQUENCE PROTOCOL

&lt;110&gt; Degussa AG

5 &lt;120&gt; New nucleotide sequences which code for the otsA gene

&lt;130&gt; 010037 BT

&lt;140&gt;

10 &lt;141&gt;

&lt;160&gt; 4

&lt;170&gt; PatentIn Ver. 2.1

15

&lt;210&gt; 1

&lt;211&gt; 3010

&lt;212&gt; DNA

&lt;213&gt; Corynebacterium glutamicum

20

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (884)..(2338)

&lt;223&gt; otsA gene

25

&lt;400&gt; 1

attgcggggc ttactgcgct gatgggttct gcgttttatt acctcttcgt tgtttattta 60

30

ggccccgtct ctgccgctgc gattgctgca acagcagttg gtttcaactgg tggtttgctt 120

gcccgtcgat tcttgattcc accgttgatt gtggcgattg ccggcatcac accaatgctt 180

ccagggtctag caatttaccg cggaatgtac gccaccctga atgatcaaac actcatgggt 240

35

ttcaccaaca ttgcggttgc tttagccact gttcatcac ttgccgctgg cgtgggtttt 300

ggtgagtgga ttgccgcgag gctacgtcgt ccaccacgct tcaaccata ccgtgcattt 360

40

accaaggcga atgagttctc cttccaggag gaagctgagc agaatcagcg ccggcagaga 420

aaacgtccaa agactaatca gagattcggg aataaaagggt aaaaatcaac ctgcttaggc 480

gtcttttcgt taaatagcgt agaatatcgg gtcgatcgct tttaaact caggaggatc 540

45

cttgccgggc aaaatcacgg aactcgtcc caccacagaa tcccttcacg ctgttgaaga 600

ggaaaccgca gccggtgccc gcaggattgt tgccacctat tctaaggact tcttcgacgg 660

50

cgtcactttg atgtgcatgc tcggcggtga acctcagggc ctgcgttaca ccaaggctcg 720

ttctgaacac gaggaagctc agccaaagaa ggctacaaag cggactcgta aggcaccagc 780

taagaaggct gctgctaaga aaacgaccaa gaagaccact aagaaaacta ctaaaaagac 840

55

caccgcaaag aagaccacaa agaagtctta agccggatct tat atg gat gat tcc 895

Met Asp Asp Ser

	aat agc ttt gta gtt gtt gct aac cgt ctg cca gtg gat atg act gtc	943
	Asn Ser Phe Val Val Val Ala Asn Arg Leu Pro Val Asp Met Thr Val	
	5 10 15 20	
5	cac cca gat ggt agc tat agc atc tcc ccc agc ccc ggt ggc ctt gtc	991
	His Pro Asp Gly Ser Tyr Ser Ile Ser Pro Ser Pro Gly Gly Leu Val	
	25 30 35	
10	acg ggg ctt tcc ccc gtt ctg gaa caa cat cgt gga tgt tgg gtc gga	1039
	Thr Gly Leu Ser Pro Val Leu Glu Gln His Arg Gly Cys Trp Val Gly	
	40 45 50	
15	tgg cct gga act gta gat gtt gca ccc gaa cca ttt cga aca gat acg	1087
	Trp Pro Gly Thr Val Asp Val Ala Pro Glu Pro Phe Arg Thr Asp Thr	
	55 60 65	
20	ggt gtt ttg ctg cac cct gtt gtc ctc act gca agt gac tat gaa ggc	1135
	Gly Val Leu Leu His Pro Val Val Leu Thr Ala Ser Asp Tyr Glu Gly	
	70 75 80	
25	ttc tac gag ggc ttt tca aac gca acg ctg tgg cct ctt ttc cac gat	1183
	Phe Tyr Glu Gly Phe Ser Asn Ala Thr Leu Trp Pro Leu Phe His Asp	
	85 90 95 100	
30	ctg att gtt act ccg gtg tac aac acc gat tgg tgg cat gcg ttt cgg	1231
	Leu Ile Val Thr Pro Val Tyr Asn Thr Asp Trp Trp His Ala Phe Arg	
	105 110 115	
35	gag gta aac ctc aag ttc gct gaa gcc gtg agc caa gtg gcg gca cac	1279
	Glu Val Asn Leu Lys Phe Ala Glu Ala Val Ser Gln Val Ala Ala His	
	120 125 130	
40	ggt gcc act gtg tgg gtg cag gac tat cag ctg ttg ctg gtt cct ggc	1327
	Gly Ala Thr Val Trp Val Gln Asp Tyr Gln Leu Leu Leu Val Pro Gly	
	135 140 145	
45	att ttg cgc cag atg cgc cct gat ttg aag atc ggt ttc ttc ctc cac	1375
	Ile Leu Arg Gln Met Arg Pro Asp Leu Lys Ile Gly Phe Phe Leu His	
	150 155 160	
50	att ccc ttc cct tcc cct gat ctg ttc cgt cag ctg ccg tgg cgt gaa	1423
	Ile Pro Phe Pro Ser Pro Asp Leu Phe Arg Gln Leu Pro Trp Arg Glu	
	165 170 175 180	
55	gag att gtt cga ggc atg ctg ggc gca gat ttg gtg gga ttc cat ttg	1471
	Glu Ile Val Arg Gly Met Leu Gly Ala Asp Leu Val Gly Phe His Leu	
	185 190 195	
60	gtt caa aac gca gaa aac ttc ctt gcg tta acc cag cag gtt gcc ggc	1519
	Val Gln Asn Ala Glu Asn Phe Leu Ala Leu Thr Gln Gln Val Ala Gly	
	200 205 210	
65	act gcc ggg tct cat gtg ggt cag ccg gac acc ttg cag gtc agt ggt	1567
	Thr Ala Gly Ser His Val Gly Gln Pro Asp Thr Leu Gln Val Ser Gly	
	215 220 225	
70	gaa gca ttg gtg cgt gag att ggc gct cat gtt gaa acc gct gac gga	1615
	Glu Ala Leu Val Arg Glu Ile Gly Ala His Val Glu Thr Ala Asp Gly	
	230 235 240	

5	agg cga gtt agc gtc ggg gcg ttc ccg atc tcg att gat gtt gaa atg	1663
	Arg Arg Val Ser Val Gly Ala Phe Pro Ile Ser Ile Asp Val Glu Met	
	245 250 255 260	
10	ttt ggg gag gcg tcg aaa agc gcc gtt ctt gat ctt tta aaa acg ctc	1711
	Phe Gly Glu Ala Ser Lys Ser Ala Val Leu Asp Leu Leu Lys Thr Leu	
	265 270 275	
15	gac gag ccg gaa acc gta ttc ctg gcc gtt gac cga ctg gac tac acc	1759
	Asp Glu Pro Glu Thr Val Phe Leu Gly Val Asp Arg Leu Asp Tyr Thr	
	280 285 290	
20	aag ggc att ttg cag cgc ctg ctt gcg ttt gag gaa ctg ctg gaa tcc	1807
	Lys Gly Ile Leu Gln Arg Leu Leu Ala Phe Glu Glu Leu Leu Glu Ser	
	295 300 305	
25	ggc gcg ttg gag gcc gac aaa gct gtg ttg ctg cag gtc gcg acg cct	1855
	Gly Ala Leu Glu Ala Asp Lys Ala Val Leu Leu Gln Val Ala Thr Pro	
	310 315 320	
30	tcg cgt gag cgc att gat cac tat cgt gtg tcg cgt tcg cag gtc gag	1903
	Ser Arg Glu Arg Ile Asp His Tyr Arg Val Ser Arg Ser Gln Val Glu	
	325 330 335 340	
35	gaa gcc gtc gcc cgt atc aat ggt cgt ttc ggt cgc atg ggg cgt ccc	1951
	Glu Ala Val Gly Arg Ile Asn Gly Arg Phe Gly Arg Met Gly Arg Pro	
	345 350 355	
40	gtg gtg cat tat cta cac agg tca ttg agc aaa aat gat ctc cag gtg	1999
	Val Val His Tyr Leu His Arg Ser Leu Ser Lys Asn Asp Leu Gln Val	
	360 365 370	
45	ctg tat acc gca gcc gat gtc atg ctg gtt acg cct ttt aaa gac ggt	2047
	Leu Tyr Thr Ala Ala Asp Val Met Leu Val Thr Pro Phe Lys Asp Gly	
	375 380 385	
50	atg aac ttg gtg gct aaa gaa ttc gtg gcc aac cac cgc gac ggc act	2095
	Met Asn Leu Val Ala Lys Glu Phe Val Ala Asn His Arg Asp Gly Thr	
	390 395 400	
55	ggc gct ttg gtg ctg tcc gaa ttt gcc gcc ggc ggc act gag ctg acc	2143
	Gly Ala Leu Val Leu Ser Glu Phe Ala Gly Ala Ala Thr Glu Leu Thr	
	405 410 415 420	
60	ggc ggc tat tta tgc aac cca ttt gat gtg gaa tcc atc aaa cgg caa	2191
	Gly Ala Tyr Leu Cys Asn Pro Phe Asp Val Glu Ser Ile Lys Arg Gln	
	425 430 435	
65	atg gtg gca gct gtc cat gat ttg aag cac aat ccg gaa tct gcg gca	2239
	Met Val Ala Ala Val His Asp Leu Lys His Asn Pro Glu Ser Ala Ala	
	440 445 450	
70	acg cga atg aaa acg aac agc gag cag gtc tat acc cac gac gtc aac	2287
	Thr Arg Met Lys Thr Asn Ser Glu Gln Val Tyr Thr His Asp Val Asn	
	455 460 465	

```

gtg tgg gct aat agt ttc ctg gat tgt ttg gca cag tcg gga gaa aac 2335
Val Trp Ala Asn Ser Phe Leu Asp Cys Leu Ala Gln Ser Gly Glu Asn
      470                      475                      480

5. tca tgaaccgcgc acgaatcgcg accataggcg ttcttccgct tgctttactg 2388
   Ser
   485

10 ctggcgctcct gtggttcaga caccgtggaa atgacagatt ccacctgggt ggtgaccaat 2448
   atttacaccg atccagatga gtcgaattcg atcagtaatc ttgtcatttc ccagcccagc 2508
   ttagattttg gcaattcttc cctgtctggt ttcactggct gtgtgccttt tacggggcgt 2568

15 gcggaattct tccaaaatgg tgagcaaagc tctgttctgg atgccgatta tgtgaccttg 2628
   tcttccttgg atttcgataa acttcccgat gattgccaa gacaagaact caaagttcat 2688
   aacgagctgg ttgatcttct gcctggttct tttgaaatct ccaggacttc tggttcagaa 2748

20 atcttgctga ctagcgatgt cgatgaactc gatcggccag caatccgctt ggtgtcctgg 2808
   atcgcgccga catcttaagg tgccagggct ttaaagtgcc aggggttctg tgggatccgt 2868

25 acactggttc ccatgacttt gactattgag gaaatcgcca agaccaaaaa gcttttggtt 2928
   gtgtccgatt ttgatggaac catcgcagga ttagcaagg acgcttacia cgttcctatc 2988
   aaccagaaat ccctcaaggc gg 3010

30
<210> 2
<211> 485
<212> PRT
35 <213> Corynebacterium glutamicum

<400> 2
Met Asp Asp Ser Asn Ser Phe Val Val Val Ala Asn Arg Leu Pro Val
  1           5           10          15
40 Asp Met Thr Val His Pro Asp Gly Ser Tyr Ser Ile Ser Pro Ser Pro
   20           25           30
45 Gly Gly Leu Val Thr Gly Leu Ser Pro Val Leu Glu Gln His Arg Gly
   35           40           45
   Cys Trp Val Gly Trp Pro Gly Thr Val Asp Val Ala Pro Glu Pro Phe
     50           55           60
50 Arg Thr Asp Thr Gly Val Leu Leu His Pro Val Val Leu Thr Ala Ser
   65           70           75           80
   Asp Tyr Glu Gly Phe Tyr Glu Gly Phe Ser Asn Ala Thr Leu Trp Pro
     85           90           95
55 Leu Phe His Asp Leu Ile Val Thr Pro Val Tyr Asn Thr Asp Trp Trp
   100          105          110

```

	His	Ala	Phe	Arg	Glu	Val	Asn	Leu	Lys	Phe	Ala	Glu	Ala	Val	Ser	Gln
		115						120					125			
5	Val	Ala	Ala	His	Gly	Ala	Thr	Val	Trp	Val	Gln	Asp	Tyr	Gln	Leu	Leu
		130					135					140				
	Leu	Val	Pro	Gly	Ile	Leu	Arg	Gln	Met	Arg	Pro	Asp	Leu	Lys	Ile	Gly
	145					150					155					160
10	Phe	Phe	Leu	His	Ile	Pro	Phe	Pro	Ser	Pro	Asp	Leu	Phe	Arg	Gln	Leu
					165					170					175	
	Pro	Trp	Arg	Glu	Glu	Ile	Val	Arg	Gly	Met	Leu	Gly	Ala	Asp	Leu	Val
				180					185					190		
15	Gly	Phe	His	Leu	Val	Gln	Asn	Ala	Glu	Asn	Phe	Leu	Ala	Leu	Thr	Gln
			195					200					205			
	Gln	Val	Ala	Gly	Thr	Ala	Gly	Ser	His	Val	Gly	Gln	Pro	Asp	Thr	Leu
20			210				215					220				
	Gln	Val	Ser	Gly	Glu	Ala	Leu	Val	Arg	Glu	Ile	Gly	Ala	His	Val	Glu
	225					230					235					240
25	Thr	Ala	Asp	Gly	Arg	Arg	Val	Ser	Val	Gly	Ala	Phe	Pro	Ile	Ser	Ile
					245					250					255	
	Asp	Val	Glu	Met	Phe	Gly	Glu	Ala	Ser	Lys	Ser	Ala	Val	Leu	Asp	Leu
				260					265					270		
30	Leu	Lys	Thr	Leu	Asp	Glu	Pro	Glu	Thr	Val	Phe	Leu	Gly	Val	Asp	Arg
			275					280					285			
	Leu	Asp	Tyr	Thr	Lys	Gly	Ile	Leu	Gln	Arg	Leu	Leu	Ala	Phe	Glu	Glu
35		290					295					300				
	Leu	Leu	Glu	Ser	Gly	Ala	Leu	Glu	Ala	Asp	Lys	Ala	Val	Leu	Leu	Gln
	305					310					315					320
40	Val	Ala	Thr	Pro	Ser	Arg	Glu	Arg	Ile	Asp	His	Tyr	Arg	Val	Ser	Arg
					325					330					335	
	Ser	Gln	Val	Glu	Glu	Ala	Val	Gly	Arg	Ile	Asn	Gly	Arg	Phe	Gly	Arg
				340					345					350		
45	Met	Gly	Arg	Pro	Val	Val	His	Tyr	Leu	His	Arg	Ser	Leu	Ser	Lys	Asn
			355					360					365			
	Asp	Leu	Gln	Val	Leu	Tyr	Thr	Ala	Ala	Asp	Val	Met	Leu	Val	Thr	Pro
50		370					375					380				
	Phe	Lys	Asp	Gly	Met	Asn	Leu	Val	Ala	Lys	Glu	Phe	Val	Ala	Asn	His
	385					390					395					400
55	Arg	Asp	Gly	Thr	Gly	Ala	Leu	Val	Leu	Ser	Glu	Phe	Ala	Gly	Ala	Ala
					405					410					415	
	Thr	Glu	Leu	Thr	Gly	Ala	Tyr	Leu	Cys	Asn	Pro	Phe	Asp	Val	Glu	Ser
				420					425					430		

Ile Lys Arg Gln Met Val Ala Ala Val His Asp Leu Lys His Asn Pro  
435 440 445

5 Glu Ser Ala Ala Thr Arg Met Lys Thr Asn Ser Glu Gln Val Tyr Thr  
450 455 460

His Asp Val Asn Val Trp Ala Asn Ser Phe Leu Asp Cys Leu Ala Gln  
465 470 475 480

10 Ser Gly Glu Asn Ser  
485

15  
<210> 3  
<211> 24  
<212> DNA  
<213> Corynebacterium glutamicum

20  
<220>  
<223> Primer otsA fwd

<400> 3  
25 cacctattct aaggacttct tcga 24

<210> 4  
<211> 22  
30 <212> DNA  
<213> Corynebacterium glutamicum

<220>  
<223> Primer otsA rev

35  
<400> 4  
accaaccagg tggaatctgt ca 22

**Patent claims**

1. An isolated polynucleotide from coryneform bacteria, comprising a polynucleotide sequence which codes for the otsA gene, chosen from the group consisting of
  - 5 a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
  - b) polynucleotide which is complementary to the  
10 polynucleotides of a) or b),
  - d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),the polypeptide preferably having the activity of  
15 trehalose 6-phosphate synthase.
2. The polynucleotide as claimed in claim 1, wherein the polynucleotide is a preferably recombinant DNA which is capable of replication in coryneform bacteria.
3. The polynucleotide as claimed in claim 1, wherein the  
20 polynucleotide is an RNA.
4. The polynucleotide as claimed in claim 2, comprising the nucleic acid sequence as shown in SEQ ID No. 1.
5. The DNA as claimed in claim 2 which is capable of replication, comprising
  - 25 (i) the nucleotide sequence shown in SEQ ID No. 1, or
  - (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or



(iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally

(iv) sense mutations of neutral function in (i).

- 5 6. The DNA as claimed in claim 5 which is capable of replication, wherein the hybridization is carried out under a stringency corresponding to at most 2x SSC.
7. The polynucleotide sequence as claimed in claim 1, which codes for a polypeptide which comprises the amino  
10 acid sequence shown in SEQ ID No. 2.
8. Coryneform bacteria in which the otsA gene is attenuated, in particular eliminated.
9. Coryneforme bacteria deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen [German Collection  
15 of Microorganisms and Cell Cultures] under no. DSM 14041.
10. A process for the fermentative preparation of L-amino acids, in particular L-lysine, wherein the following steps are carried out:
- 20 a) fermentation of the coryneform bacteria which produce the desired L-amino acid and in which at least the otsA gene or nucleotide sequences which code for it are attenuated, in particular eliminated;
- 25 b) concentration of the L-amino acid in the medium or in the cells of the bacteria, and
- c) isolation of the L-amino acid.
11. The process as claimed in claim 10, wherein a Corynebacterium glutamicum strain deposited at the  
30 Deutsche Sammlung für Mikroorganismen und Zellkulturen

[German Collection of Microorganisms and Cell Cultures]  
under no. DSM 14041 is employed.

12. The process as claimed in claim 10, wherein bacteria in  
which further genes of the biosynthesis pathway of the  
desired L-amino acid are additionally enhanced are  
employed.
13. The process as claimed in claim 10, wherein bacteria in  
which the metabolic pathways which reduce the formation  
of the desired L-amino acid are at least partly  
eliminated are employed.
14. The process as claimed in claim 10, wherein the  
expression of the polynucleotide(s) which code(s) for  
the otsA gene is attenuated, in particular eliminated.
15. The process as claimed in claim 10, wherein the  
regulatory (or catalytic) properties of the polypeptide  
(enzyme protein) for which the polynucleotide otsA  
codes are reduced.
16. The process as claimed in claim 10, wherein for the  
preparation of L-amino acids, coryneform microorganisms  
are fermented in which at the same time one or more of  
the genes chosen from the group consisting of
- 16.1 the dapA gene which codes for dihydrodipicolinate  
synthase,
- 16.2 the gap gene which codes for glyceraldehyde 3-  
phosphate dehydrogenase,
- 16.3 the eno gene which codes for enolase,
- 16.4 the tpi gene which codes for triose phosphate  
isomerase,
- 16.5 the pgk gene which codes for 3-phosphoglycerate  
kinase,

- 16.6 the zwf gene which codes for glucose 6-phosphate dehydrogenase,
- 16.7 the pyc gene which codes for pyruvate carboxylase,
- 5 16.8 the mqo gene which codes for malate-quinone oxidoreductase,
- 16.9 the lysC gene which codes for a feed-back resistant aspartate kinase,
- 16.10 the lysE gene which codes for lysine export,
- 10 16.11 the zwal gene which codes for the Zwal protein is or are enhanced or over-expressed.
17. The process as claimed in claim 10, wherein for the preparation of L-amino acids, coryneform microorganisms are fermented in which at the same time one or more of
- 15 the genes chosen from the group consisting of
- 17.1 the pck gene which codes for phosphoenol pyruvate carboxykinase,
- 17.2 the pgi gene which codes for glucose 6-phosphate isomerase,
- 20 17.3 the poxB gene which codes for pyruvate oxidase,
- 17.4 the zwa2 gene which codes for the Zwa2 protein,
- 17.5 the fda gene which codes for fructose 1,6-bisphosphate aldolase,
- 25 17.6 the hom gene which codes for homoserine dehydrogenase
- 17.7 the thrB gene which codes for homoserine kinase,

17.8 the panD gene which codes for aspartate  
decarboxylase

is or are attenuated.

- 5 18. Coryneform bacteria which contain a vector which  
carries parts of the polynucleotide as claimed in claim  
1, but at least 15 successive nucleotides of the  
sequence claimed.
- 10 19. The process as claimed in one or more of the preceding  
claims, wherein microorganisms of the species  
Corynebacterium glutamicum are employed.
- 15 20. A process for identifying RNA, cDNA and DNA in order to  
isolate nucleic acids or polynucleotides or genes which  
code for trehalose 6-phosphate synthase or have a high  
similarity with the sequence of the otsA gene, wherein  
the polynucleotide comprising the polynucleotide  
sequences as claimed in claims 1, 2, 3 or 4 is employed  
as hybridization probes.
21. Process as claimed in claim 20, wherein arrays, micro  
arrays or DNA chips are employed.

**Abstract**

The invention relates to an isolated polynucleotide comprising a polynucleotide sequence chosen from the group consisting of

- 5 a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- b) polynucleotide which codes for a polypeptide which  
10 comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- c) polynucleotide which is complementary to the polynucleotides of a) or b), and
- 15 d) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b) or c),

and a process for the fermentative preparation of L-amino acids using coryneform bacteria in which at least the otsA  
20 gene is present in attenuated form, and the use of polynucleotides which comprise the sequences according to the invention as hybridization probes.

Figure 1: Plasmid pUC18otsA

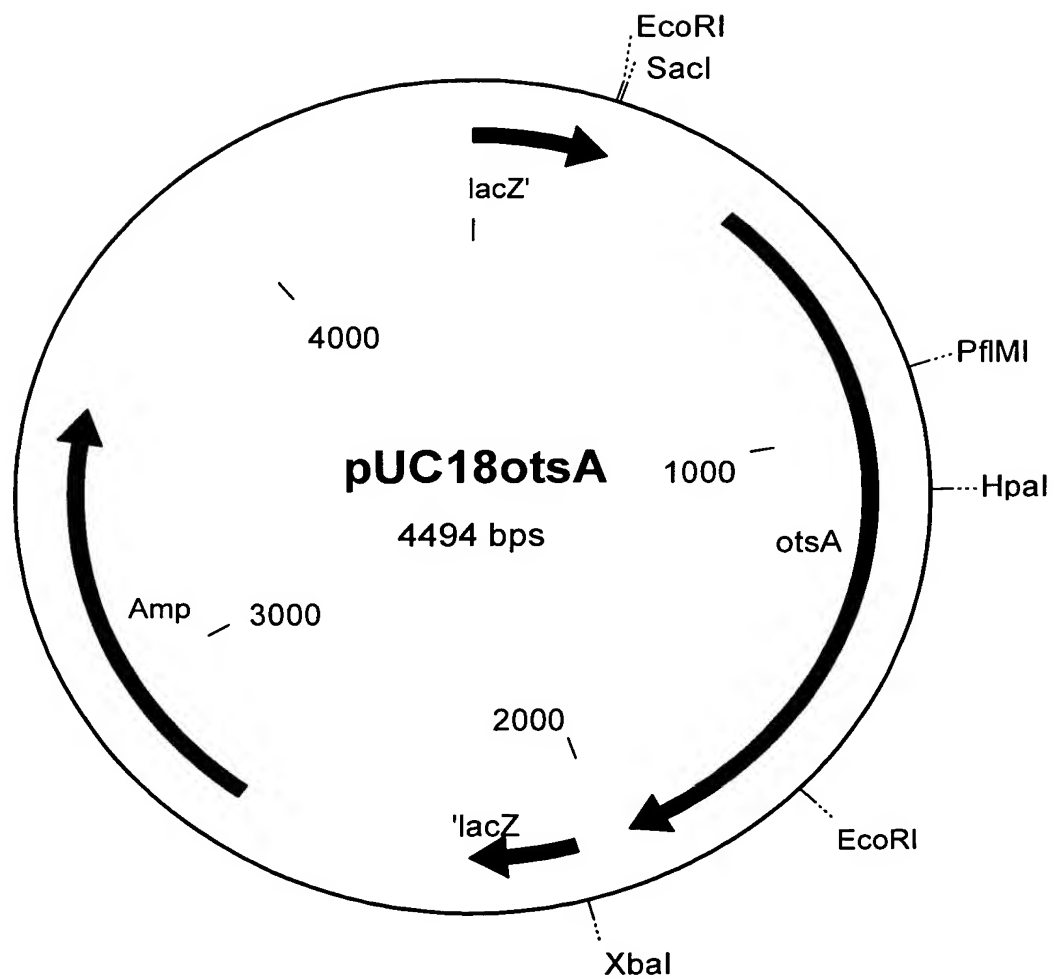


Figure 2: Plasmid pK19mobsacB $\Delta$ otsA